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14. ABSTRACT

The goal of this work was to develop a model of a realistic polymicrobial infection with bony involvement in an internally stabilized segmental defect in the rat femur. This model could then be used to assess the combined therapy of an osteogenic agent to stimulate bone formation while local and systemic antibiotic therapy was being applied to control the polymicrobial infection. A bone isolate of *Acinetobacter baumannii* exhibited very little osteolytic involvement when used alone in the model. Qualitative cultures indicated very little *A. baumannii* in the defect after contamination, but quantitative bacteriology showed *A. baumannii* residing within the bone at levels 3 to 4 logs less than the contaminating inoculum. Assessments in the polymicrobial model suggest that the osteolytic effect of *S. aureus* was not significantly amplified by the presence of the *A. baumannii*. Quantitative bacteriology revealed that *A. baumannii* was still recovered from the femur, and levels of *S. aureus* were similar to when *S. aureus* was used alone. In summary, we were unable to obtain a robust enough polymicrobial infection with bony involvement when using *S. aureus* and *A. baumannii*. After consultations with military collaborators, we will repeat the experiment replacing *Acinetobacter* with *Pseudomonas aeruginosa*.

15. SUBJECT TERMS
segmental defect, chronic infection, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, antibiotic, osteomyelitis, debridement, rat model

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Introduction

The majority of the combat casualties in Operations Iraqi Freedom and Enduring Freedom are a result of high-energy blast or high-velocity projectile mechanisms, and commonly present with a significant segmental bone defect, massive soft tissue disruption, and substantial contamination with bacteria. The goal of this study was to develop a model of a polymicrobial chronic infection in an internally-stabilized segmental defect in the rat femur. The model will then be used to study the effect of debridement and antibiotic therapy in the treatment of this polymicrobial chronic infection. This study consisted of four specific aims. An initial screening was performed in Aim #1 to determine a contaminating inoculum of *Acinetobacter baumannii* and a time from contamination that would reliably produce an infection in an internally-stabilized segmental defect, yet not cause the animals to become septic, and not cause a significant amount of bony lysis that would seriously compromise defect fixation. The objective of Aim #2 was to assess the effectiveness of treatment of this chronic infection by surgical debridement with and without local antibiotic therapy with gentamicin introduced in a ceramic-collagen matrix carrier. Aim #3 involved repeating Aim #1 except that combinations of inocula of *A. baumannii* and *S. aureus* and times from contamination were screened. Finally, the goal of Aim #4 was to treat this polymicrobial infection with debridement with and without local administration of gentamicin. This study will provide direct translational information to optimize the use of local antibiotics and commercially available bone graft materials or carriers to deliver these antibiotics, for improved treatment of infected segmental bone loss which frequently occurs in combat casualties.

Body

Aim #1: To summarize our initial screening work, we were unable to obtain a clinical infection with any observable bony lysis involvement in our segmental defect model in the rat after contamination with a range of inocula (10^4 to 10^8 colony forming units (CFUs)) of *Acinetobacter baumannii* from both tracheal and bone isolates. We finalized Aim #1 by obtaining a complete set of publishable data to confirm what we observed with our initial screening work with *A. baumannii* alone in the segmental defect. The plan in Table 1 below was followed.

Table 1. Study design for Aim #1

Intervention		Time from contamination		
		2 weeks	4 weeks	8 weeks
A. baumannii from tracheal isolate: 10^8 CFUs		10*	10	10
A. baumannii from bone isolate: 10^8 CFUs		10	10	10

* 10 animals at each intervention and time point, with 5 used for quantitative bacterial cultures, and 5 for high-resolution radiographic lysis assessments. Qualitative cultures were also taken under sterile conditions at the time that all animals were euthanized.

The surgeries on the 60 animals in Table 1 were completed, all animals in both isolate groups

were euthanized at 2, 4 or 8 weeks after contamination, and all assessments were completed. The findings are as follows.

Bony Lysis: High-resolution Faxitron radiographs of the femurs with defect were obtained at 2, 4 and 8 weeks after contamination. It has been previously demonstrated in this model that bony lysis, if it occurs, first becomes radiographically evident where the K-wires cross the cortical bone.[1] Bony lysis was assessed by simply counting the number of these locations where lysis occurred (12 possible sites of lysis where the 6 K-wires cross the cortical bone twice). This number of sites of lysis has been shown in our previous work to significantly correlate with the torsional stiffness of the defect fixation with the plate and K-wires.

Very little (if any) bony lysis was evident from the high-resolution radiographs at 2 to 8 weeks when the defects were contaminated with 10^8 CFUs of *A. baumannii* from both tracheal and bone isolates (Table 2). There were no significant differences in the median numbers of sites of lysis between any of the time points and interventions (ANOVA on ranks). *A. baumannii* did not exhibit bony lysis involvement radiographically in this model. However, 10 of the 15 femurs contaminated with 10^8 CFUs of *A. baumannii* from the bone isolate exhibited some newly formed bone capping the ends of the defect (Figure 1). Similarly, 10 of the 15 femurs contaminated with 10^8 CFUs of *A. baumannii* from the tracheal isolate also exhibited some newly formed bone capping the ends of the defect, and this new bone formation nearly connected the ends of the defect in 2 of these 10 animals (Figure 2). This has never been previously observed in our model without some form of osteogenic treatment. One explanation could be that the newly formed bone may be due to the presence of the absorbable collagen sponge used to retain the bacteria in the defect in the short term. However, we have previously shown that the presence of the collagen sponge in the uninfected defect was not osteogenic in and of itself.[2-4] And, we have also previously shown that our defect model is critical – that no bone forms unless an osteogenic treatment is applied.[2-4] We do not regard this information as definitive, but it brings up the question for further study and confirmation as to whether the *A. baumannii* somehow plays a role in a cascade of events that results in new bone formation.

Table 2. Number of sites of bony lysis from high resolution radiographs*

Intervention	Time from Contamination		
	2 weeks	4 weeks	8 weeks
A. baumannii from tracheal isolate: 10^8 CFUs	0 (0.50)	0 (0.25)	0 (0)
A. baumannii from bone isolate: 10^8 CFUs	0 (1.25)	0 (0.25)	0 (1.25)

* data shown as median (interquartile range)

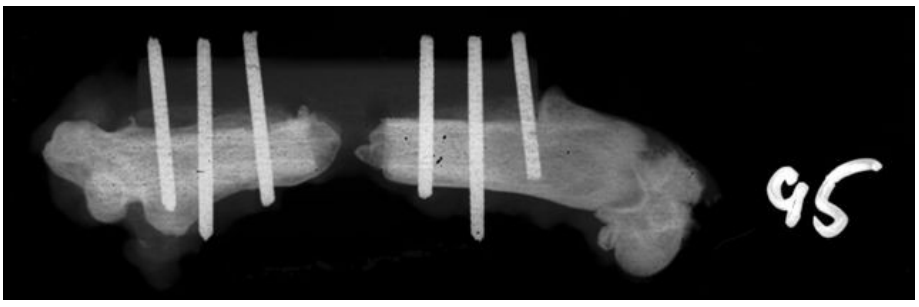

Figure 1. Example of bone capping the end of the defect contaminated with *A. baumannii*

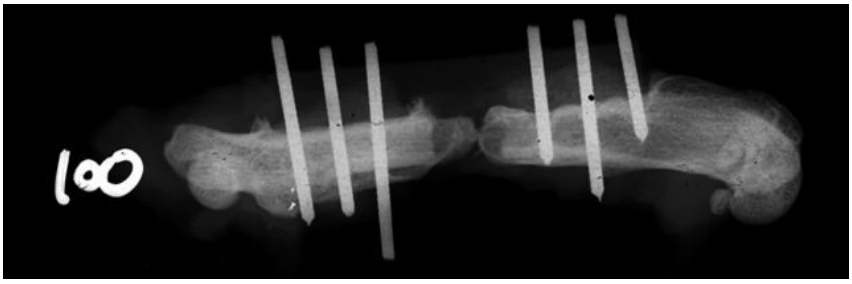

Figure 2. Example of bone nearly connecting the ends of the defect contaminated with *A. baumannii*

Qualitative Bacteriology: Bacterial swabs of the defects taken at the time the animals were euthanized revealed the presence of very few (if any) colonies of *A. baumannii*, as summarized in Table .

Table 3. Results of qualitative bacteriology for *A. baumannii*

Culture Results	Bone Isolate			Tracheal Isolate		
	2 wk	4 wk	8 wk	2 wk	4 wk	8 wk
No growth	6	8	9	8	10	10
Rare	1	1		2		
Few	2		1			
Moderate						
Many						
Contaminated*	1	1				

* Contamination with another type of bacteria - no *A. baumannii* was isolated

Quantitative Bacteriology: The means of the numbers ($\log_{10}$) of CFUs of *A. baumannii* recovered from the contaminated femurs are summarized in Table 4 below. The numbers of bacteria recovered from the bone are greater than would be expected, given the results of the cultures from the bacterial swabs in Table 3. However, the mean numbers of recovered CFUs of *A. baumannii*, both from the bone and tracheal isolates, were 3 to 4 logs less than the original contaminating inoculum. This finding suggests that the host's immune system adequately deals with most of the bacteria, but that some bacteria still exist within the bone.

Table 4. Number of CFUs ($\log_{10}$) of *A. baumannii* recovered from contaminated femurs*

Intervention	Time from Contamination		
	2 week	4 week	8 week
A. baumannii from tracheal isolate: 10^8 CFUs	5.11 (1.70-5.58)	5.16 (3.70-5.59)	5.56 (0-6.08)
A. baumannii from bone isolate: 10^8 CFUs	4.53 (2.93-5.09)	5.04 (4.11-5.35)	3.62† (0-4.30)

* data shown as the mean (range) of 5 samples

† significantly less than with a bone isolate 4 weeks (ANOVA on ranks, Dunn's method for pairwise testing, $p < 0.05$)

Summary of Aim #1 Results: Both bone and tracheal isolates of *A. baumannii* exhibited very little osteolytic bony involvement in our model. Evidence of new bone formation occurred at the ends of the defect with contamination with *A. baumannii* without introduction of any osteogenic agent. Qualitative cultures from bacterial swabs indicated very little *A. baumannii* in the defect after contamination. However, quantitative bacteriology revealed on the order of 10^4 to 10^5 CFUs of *A. baumannii* recovered from the femur, although this was 3 to 4 logs less than the contaminating inoculum. These results suggest that the *A. baumannii* resides within the bone in this model, which imitates what actually happens clinically. However, we were looking for more bony involvement since we will eventually use this model to assess the ability of an osteogenic agent to heal the defect and counteract bony lysis while local/systemic antibiotic therapy overcomes the infection as the fixation implant is left in place.

Aim #3: After consultation with the Program Director of OTRP, Dr. Josh Wenke, and with Dr. Clinton Murray from Brooke Army Medical Center regarding the lack of a robust infection with bony involvement from *A. baumannii* in our model, our team received permission from OTRP to omit the originally proposed Aim #2 and proceed directly to assessment of a polymicrobial infection with *S. aureus* and *A. baumannii*. Drs. Wenke and Murray suggested that we may want to consider substituting *Klebsiella* or *Pseudomonas aeruginosa* for *Acinetobacter*, because these bacteria are also problematic in severe combat wounds. However, we chose to remain with *A. baumannii* as one of the polymicrobial bacteria because we have already invested significant time in using it in our model, and would need to repeat the evaluation of another new bacteria alone in the model if we change at this point. The study design in Table 5 below was followed.

The goal of Aim #3 was to perform a screening to determine contaminating inocula of *A. baumannii* and *S. aureus* and a time from contamination that would reliably produce an infection in our internally-stabilized segmental defect model, yet not cause the rats to become septic, and not cause a significant amount of bony lysis that would seriously compromise defect fixation. We began by using the "optimal" inocula for the two bacteria determined to date: 10^8 CFUs of *A. baumannii* from the bone isolate (the largest inoculum we could reliably prepare) and 10^4 CFUs of *S. aureus* (from our previous published studies[1-4]), and then bracket down the *S. aureus* to 10^3 CFUs for a second intervention (we kept the 10^8 CFU *A. baumannii* inoculum since it still did not produced an overwhelming response, even when coupled with *S. aureus* in the polymicrobial setting). The surgeries and assessments of these polymicrobial animals have been completed, and our findings are as follows.

Table 5. Study design for Aim #3

Intervention		Time from Contamination		
		1 week	2 week	3 week
S. aureus: 10^4 CFUs *A. baumannii*: (bone isolate): 10^8 CFUs		10*	10	10
S. aureus: 10^3 CFUs *A. baumannii*: (bone isolate): 10^8 CFUs		10	10	10

* 10 animals at each intervention and time point, with 5 used for quantitative bacterial cultures, and 5 for high resolution radiographic lysis assessments. Cultures were also taken under sterile conditions at the time that all animals are euthanized.

Bony Lysis: Bony lysis resulting from the polymicrobial contamination was generally greater than with *A. baumannii* alone (Aim #1), although there was large variability over the animals tested (Table 6). There were no significant differences in lysis between the interventions and time points (ANOVA on ranks). Lysis with the polymicrobial contamination was comparable to that with *S. aureus* alone in our previous work,[1] although again, the variability among the animals tested in this present study was greater. No consistent new bone formation in the defect was observed as with *A. baumannii* alone (Figure 4). This lysis assessment suggests that the osteolytic effect of *S. aureus* was not significantly amplified by the *A. baumannii*.

Table 6. Number of sites of bony lysis from high resolution radiographs*

Intervention	**1 week**	**2 week**	**3 week**
S. aureus: 10^4 CFUs *A. baumannii*: (bone isolate): 10^8 CFUs	2 (3.75)	0 (.25)	2 (2.25)
S. aureus: 10^3 CFUs *A. baumannii*: (bone isolate): 10^8 CFUs	0 (0.25)	0 (0)	2 (2.25)

* data shown as median (interquartile range)

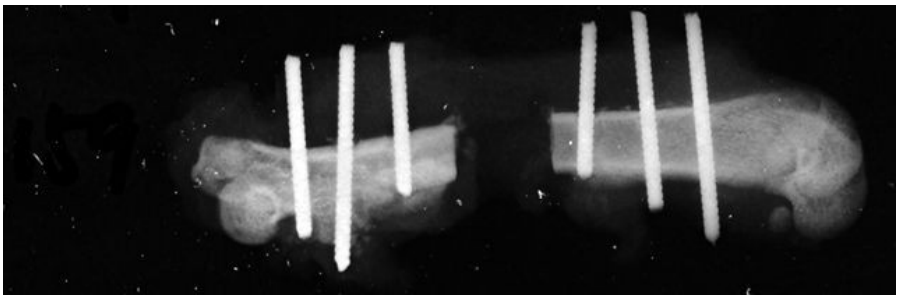

Figure 4. Radiograph of femur with defect at 1 week after contamination with 10^4 CFUs of *S. aureus* and 10^8 CFUs of *A. baumannii* from the bone isolate (note the sites of lysis)

Qualitative Bacteriology: Bacterial swabs of the polymicrobial-contaminated defects taken at the time the animals were euthanized revealed very little if any *A. baumannii* at the defect site, even though the *S. aureus* was present (Table 7). As expected and confirming our previous work with this model,[1] cultures revealed a high prevalence of *S. aureus* from 1 to 3 weeks.

Table 7. Results of qualitative bacteriology for polymicrobial model

Culture Results	10^4 *S. aureus* + 10^8 *A. baumannii*						10^3 *S. aureus* + 10^8 *A. baumannii*					
	1 wk		2 wks		3 wks		1 wk		2 wks		3 wks	
	Ab*	Sa*	Ab	Sa	Ab	Sa	Ab	Sa	Ab	Sa	Ab	Sa
No growth	3		9	1	9		8		10		10	
Rare	4		1				2					
Few	3			1								
Moderate												
Many		10		8		9		10		10		10

* Ab = *A. baumannii*, Sa = *S. aureus*

Quantitative Bacteriology: The numbers of CFUs of *A. baumannii* and *S. aureus* recovered from the femurs with a polymicrobial contamination are summarized in Table 8 below. The numbers of recovered *A. baumannii* remained at a level 3 to 4 logs less than the contamination level of 10^8 CFUs, and were not substantially different than levels without *S. aureus* in Table 4. The numbers of recovered *S. aureus* were 2 to 5 logs greater than their contamination levels of 10^3 and 10^4 CFUs, and were similar to levels in our previously published work for *S. aureus* alone.[1] Greater levels of both bacteria were recovered when the polymicrobial contamination was 10^3 *S. aureus* + 10^8 *A. baumannii*, compared with 10^4 *S. aureus* + 10^8 *A. baumannii*.

Table 8. Number of CFUs (Log_{10}) of *A. baumannii* (Ab) and *S. aureus* (Sa) recovered from contaminated femurs*

Polymicrobial Contamination Condition	Time from Contamination					
	1 wk		2 wks		3 wks	
	Ab*	Sa*	Ab	Sa	Ab	Sa
10^4 *S. aureus* + 10^8 *A. baumannii*	4.63 (2.95-4.95)	6.90 (6.43-7.22)	4.57 (4.31-4.79)	6.97§ (6.53-7.26)	4.56 (0-5.00)	6.02 (4.30-6.44)
10^3 *S. aureus* + 10^8 *A. baumannii*	5.53 (4.49-6.00)	7.94† (7.10-8.30)	5.16 (4.30-5.63)	7.47 (7.00-7.83)	5.26 (4.62-5.42)	7.03 (6.38-7.40)

* data shown as the mean (range) of 5 samples

† significantly greater than *A. baumannii* at 1, 2 and 3 weeks (ANOVA on ranks, Dunn's method for pairwise testing, $p < 0.05$)

§ significantly greater than *A. baumannii* at 1 and 3 weeks (ANOVA on ranks, Dunn's method for pairwise testing, $p < 0.05$)

Summary of Aim #3 Results: The lysis assessment suggests that the osteolytic effect of *S. aureus* was not significantly amplified by the presence of the *A. baumannii*. There was no consistent new bone formation in the defect with the polymicrobial infection as was observed as with *A. baumannii* alone; the *S. aureus* apparently inhibited the bone-forming mechanism that was stimulated by *A. baumannii* when present by itself. Qualitative cultures from bacterial swabs indicated very little *A. baumannii* in the defect after polymicrobial contamination, just as with contamination with *A. baumannii* alone. Quantitative bacteriology revealed the presence of 10^4 to 10^5 CFUs of *A. baumannii* recovered from the femur, although again this was 3 to 4 logs less than the contaminating inoculum. The levels of *S. aureus* were 2 to 5 logs greater than the contaminating inocula, and did not appear to be amplified by the presence of the *A. baumannii*. Findings in Aim #3 confirmed previously reported results using this model with *S. aureus* alone.[1] As in Aim #1, we did not find the expected synergistic effect with enhanced bony involvement.

Future Work: We were unable to obtain a robust enough polymicrobial infection with bony involvement when using *S. aureus* and *A. baumannii*. Bony lysis involvement is important because this model will eventually be used to assess the combined therapy of an osteogenic agent to stimulate bone formation while local and systemic antibiotic were being used to control the polymicrobial infection. After review of the literature and consultations with Drs. Wenke and

Murray, as well as Dr. Glenn Wortmann, an infectious disease specialist from Walter Reed Medical Center, we received permission from OTRP to replace *Acinetobacter* with *Pseudomonas aeruginosa* in our polymicrobial infection model. We will repeat screening work with the new bacteria alone (Aim #1) and in combination with *S. aureus* (Aim #3) before proceeding with a treatment arm of the study (Aim #4). We are in the process of obtaining a suitable bone isolate of *P. aeruginosa* from a war wound from Dr. Murray. **We asked for and were granted a no-cost extension for this work into 2008.** The following experimental designs will be followed.

Repeat Aim #1. Determine the appropriate inoculum of *Pseudomonas aeruginosa* and time from contamination that will consistently create an infection

Table 9. Study design for Repeat Aim #1

Inoculum of *P. aeruginosa* (CFUs)		Time from contamination	
		2 weeks	3 weeks
10^6		6*	6
10^5		6	6
10^4		6	6
10^3		6	6

* 6 animals at each intervention and time point: plane radiographs will first be taken to assess evidence of osteomyelitis, and then bacterial census measurements will subsequently be made. Cultures will also be taken under sterile conditions at the time that all animals are euthanized.

Repeat Aim #3. Determine the appropriate inocula of *Pseudomonas aeruginosa* and *Staphylococcus aureus,* and time from contamination that will consistently create an infection

Table 10. Study design for Repeat Aim #3

Intervention		Time from contamination	
		2 weeks	3 weeks
S. aureus: 10^4 CFUs *Pseudomonas aeruginosa*: 10^3 CFUs		6	6
S. aureus: 10^4 CFUs *Pseudomonas aeruginosa*: 10^4 CFUs		6	6
S. aureus: 10^4 CFUs *Pseudomonas aeruginosa*: 10^6 CFUs		6	6
S. aureus: 10^3 CFUs *Pseudomonas aeruginosa*: 10^3 CFUs		6	6
S. aureus: 10^3 CFUs *Pseudomonas aeruginosa*: 10^4 CFUs		6	6
S. aureus: 10^3 CFUs *Pseudomonas aeruginosa*: 10^6 CFUs		6	6

* 6 animals at each intervention and time point: plain radiographs will first be taken to reveal evidence of osteomyelitis, and then bacterial census measurements will subsequently be made. Cultures will also be taken under sterile conditions at the time that all animals are euthanized.

This will be followed by the treatment arm of the study (Aim #4).

Key Research Accomplishments

- Development and characterization of an animal model of an internally-stabilized segmental defect in the rat femur with a chronic infection from *Acinetobacter baumannii*.

- Using the above model, we learned that …
  - ► both bone and tracheal isolates of *A. baumannii* exhibited very little osteolytic bony involvement.
  - ► qualitative cultures from bacterial swabs indicated very little *A. baumannii* in the defect after contamination.
  - ► on the order of 10^4 to 10^5 CFUs of *A. baumannii* were recovered from the femur using quantitative bacteriology, although this was 3 to 4 logs less than the contaminating inoculum; the remaining *A. baumannii* appears to reside within the bone.
  - ► there was evidence of new bone formation occurring at the ends of the defect with *A. baumannii* contamination, without introduction of any osteogenic agent; this suggests that *A. baumannii* somehow plays a role in a cascade of events which results in new bone formation.

- Development and characterization of an animal model of an internally-stabilized segmental defect in the rat femur with a polymicrobial chronic infection from *Acinetobacter baumannii* and *Staphylococcus aureus*.

- Using the above polymicrobial model, we learned that …
  - ► the osteolytic effect of *S. aureus* was highly variable and not significantly amplified by the presence of the *A. baumannii*; lysis with the polymicrobial contamination was comparable to that with *S. aureus* alone in our previous work.
  - ► qualitative cultures from bacterial swabs showed very little *A. baumannii* in the defect, similar to contamination with *A. baumannii* alone.
  - ► 10^4 to 10^5 CFUs of *A. baumannii* were recovered from the femur using quantitative bacteriology, although again this was 3 to 4 logs less than the contaminating inoculum; the levels of *S. aureus* were 2 to 5 logs greater than the contaminating inocula, and did not appear to be amplified by the presence of the *A. baumannii*.
  - ► there was no consistent new bone formation in the defect as was observed with *A. baumannii* alone; the *S. aureus* apparently inhibited the bone-forming mechanism that was stimulated by *A. baumannii* when present by itself.

Reportable Outcomes

- Animal model of an internally-stabilized segmental defect in the rat femur with a chronic infection from *Acinetobacter baumannii*
- Animal model of an internally-stabilized segmental defect in the rat femur with a

polymicrobial chronic infection from *Acinetobacter baumannii* and *Staphylococcus aureus*

- Manuscripts, abstracts and presentations will be forthcoming as the project is finished during the grant extension period, as well as animal models similar to the two above but with *Pseudomonas aeruginosa* instead of *Acinetobacter baumannii*.

Conclusions

The goal of this work was to develop a model of a polymicrobial infection with bony involvement that could be used to assess the combined therapy of an osteogenic agent to stimulate bone formation and counteract bony lysis, while local and systemic antibiotic were being used to control the polymicrobial infection while the fixation implant was left in place.

Both bone and tracheal isolates of *A. baumannii* exhibited very little osteolytic bony involvement when used alone in our model. Qualitative cultures indicated very little *A. baumannii* in the defect after contamination. However, quantitative bacteriology showed that 10^4 to 10^5 CFUs of *A. baumannii* were recovered from the femur, although this was 3 to 4 logs less than the contaminating inoculum. These results suggest that the *A. baumannii* resided within the bone in this model, which imitates what actually happens clinically. However, we were looking for more bony involvement.

Assessments in the polymicrobial model suggest that the osteolytic effect of *S. aureus* was not significantly amplified by the presence of the *A. baumannii*. Qualitative cultures from bacterial swabs indicated very little *A. baumannii* in the defect after polymicrobial contamination, just as with contamination with *A. baumannii* alone. Quantitative bacteriology revealed the presence of 10^4 to 10^5 CFUs of *A. baumannii* recovered from the femur, although again this was 3 to 4 logs less than the contaminating inoculum. The levels of *S. aureus* were 2 to 5 logs greater than the contaminating inocula, and were similar to levels in our previously published work for *S. aureus* alone.[1] As with Aim #1, we did find the expected synergistic effect with enhanced bony involvement.

In many of the defects contaminated with 10^8 CFUs of *A. baumannii* from the bone or tracheal isolates, newly formed bone was found to cap the ends of the defect, and in some animals nearly connected the ends of the defect. This has never been previously observed in our model without some form of osteogenic treatment. We have shown that the presence of the collagen sponge in the uninfected defect was not osteogenic in and of itself.[2-4] And, we have also previously shown that our defect model is critical – that no bone forms unless an osteogenic treatment is applied.[2-4] This leads us to suspect that the *A. baumannii* somehow plays a role in a cascade of events that results in new bone formation. There was no consistent new bone formation in the defect with the polymicrobial infection as was observed as with *A. baumannii* alone. The *S. aureus* apparently inhibited the bone-forming mechanism that was stimulated by *A. baumannii* when present by itself.

In summary, we were unable to obtain a robust enough polymicrobial infection with bony involvement when using *S. aureus* and *A. baumannii*. Bony involvement is important because this model will eventually be used to assess the combined therapy of an osteogenic agent to stimulate bone formation while local and systemic antibiotic were being used to control the

polymicrobial infection. After review of the literature and consultations with military collaborators, we received permission from OTRP to replace *Acinetobacter* with *Pseudomonas aeruginosa* in our polymicrobial infection model. This will hopefully give us a model more clinically relevant to the combat trauma we are trying to better treat. We will repeat screening work with the new bacteria alone (Aim #1) and in combination with *S. aureus* (Aim #3) before proceeding with a treatment arm of the study (Aim #4). We asked for and were granted a no-cost extension for this work into 2008.

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Personnel Receiving Pay From the Grant

Dean T. Tsukayama, MD: Principal Investigator

Joan E. Bechtold, PhD: Co-Investigator

David W. Polly Jr, MD: Co-Investigator

William D. Lew, MS: Investigator

Carlos A. Castro, MD: Animal surgeon

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Brooke Sommer: Animal care technician

Appendices

No entries

Supporting Data

All supporting data is embedded in the body of the report